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## Determination of Hexuronic Acids in Fruit by Gas Chromatography<sup>1,2</sup>

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**Abstract.**  $\alpha$ -D-galacturonic acid,  $\beta$ -D-glucuronic and  $\alpha$ -D-glucose and  $\alpha$ -L-arabinose were separated simultaneously by gas liquid chromatography as the trimethylsilyl (TMS) derivatives made by the direct addition of dry pyridine, hexamethyldisilazane, and trimethylchlorosilane (9:3:1) to uronic acids and sugars at room temperatures. Useful for determination of fruit cell-wall constituents, the method is simple, fast and the TMS derivatives appear directly related to the concentrations of hexuronic acids in the reaction mixtures. Anomerization of the uronic acids was very difficult to avoid in experimental samples. The  $\beta$ -D-TMS-galacturonic acid was separated from  $\beta$ -D-TMS-glucuronic acid but the  $\alpha$ -D-TMS-galacturonic acid was not separated from the  $\alpha$ -D-TMS-glucuronic acid on the SE-30 column used. The substance referred to as  $\gamma$ -D-TMS-galacturonic acid, although not identified, was found in acid mutarotation mixtures of D-galacturonic acid and also in uronic acids extracted from apple tissue. The measurement of the anomers of D-galacturonic acid from apple tissue was made by enzymatic degradation of the pectic substances, separation of the hydrolytic products on paper chromatograms, eluting the uronic acids, and treating the dried uronic acids with the pyridine-silane mixture.

### INTRODUCTION

THE measurement of hexuronic acids which are considered the basic constituents of the pectic substances of fruit cell-walls has long presented difficulties. One must agree that the pectic substances are mainly poly-galacturonic acid chains and must be determined either as the polymer or the monomer. The latter determination is preferred simply from the standpoint of ease and precision of measurement; however, it is extremely difficult to reconstruct conditions as they were initially in the plant cell-walls. Difficulty in extracting pure polysaccharides, lack of quantitative methods to ascertain small quantities of the hexuronides in complex mixtures and poor correlations between the pectic substances and measurable changes in texture of apples and other fruits and vegetables are continuing to plague workers in the field.

Ash and Reynolds (2) and Tavakoli and Wiley (10) have indicated difficulties in separating hexuronic acids in apple fruit by paper partition chromatography, using both washed and unwashed papers and a variety of solvents. Helbut and Brown (6), however, have reported a method of measuring both galacturonic and glucuronic acids by use of the anthrone- $H_2SO_4$  method.

Recently, by use of gas chromatography, great strides have been made in the separation of carbohydrates and related polyhydroxy compounds. McInnes *et al.* (7) reported a classic study on the sepa-

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ration of tri-O-methyl derivatives of methyl pentopyranosides and tetra-O-methyl derivatives of hexopyranosides by this technique. Hedgley and Overend (5) and Ferrier and Singleton (4) described conditions for the preparation and determination of trimethylsilyl (TMS) derivatives of a number of carbohydrates. Bentley *et al.* (3), Sweeley *et al.* (9), and Sweeley and Walker (8) reported the relative retention time of more than 100 carbohydrates as TMS derivatives. The separation was made after conversion of sugars to methyl glycosides and then conversion of the free hydroxyl groups in the monomeric carbohydrates to TMS derivatives. They concluded that these derivatives are ideally suited for routine determinations.

A search of available literature has not revealed the separation and measurement of aldohexuronic acids as TMS derivatives. The present study describes the development of a method to measure hexuronic acids alone and in complex mixtures. Separation of glucose and arabinose from the uronic acids and the special preparation of hexuronic acids from apple tissue and their separation are also described. This work to improve measurements of hexuronic acids should aid in studies of maturity and ripening changes in fruit, modifications in texture during processing and better utilization of cell-wall products in the nutrition of humans.

#### MATERIALS AND METHODS

*Apparatus:* Gas chromatograph measurements were made with an Aerograph 200 series (Model 204) equipped with a hydrogen flame detector and a stainless steel  $5' \times \frac{1}{8}"$  OD column packed with SE-30, 5% silicone polymer on acid washed 60–80 mesh chromosorb W. Except for the mutarotated uronic acids, the operating conditions were: column oven temperature, 178° C; injection temperature, 200° C; detector temperature, 178° C; N<sub>2</sub> carrier gas flow 20 ml/min.; hydrogen flow 20 ml/min. and air flow 250 ml/min. A Leeds and Northrup Speed-O-Max G strip recorder with a speed of 1"/min. was used. Samples for injection were measured with a 5 $\mu$ l Hamilton micro-syringe.

*Reagents and Standards:* The TMS derivatives were supplied by Applied Science Laboratories Inc., State College, Pennsylvania. One ml ampoules containing dry pyridine, hexamethyldisilazane, trimethylchlorosilane (9:3:1 v/v) were used to convert the free carbohydrates to silyl ethers. Not more than 20 mg/ml of the hexuronide was used in the development of standard curves.

The  $\alpha$ -D-galacturonic acid standard, 99% pure, and the  $\beta$ -D-glucuronic acid standard, 97–99% pure, were supplied by K & K Laboratories Inc., Plainview, New York. They were kept dry in desiccators at 20° C prior to the addition of silanes. The galacturonic and glucuronic acids were mutarotated in H<sub>2</sub>O at room temperature, at pH 3.2 and at pH 5.5 for 24 hours.

The  $\alpha$ -L-arabinose and  $\alpha$ -D-glucose were obtained from Fisher Scientific, Silver Spring, Maryland.

*Separation of apple hexuronic acids:* The apple hexuronic acids were separated as suggested by Tavakoli and Wiley (10). The main

opportunity for mutarotation of the galacturonic acid occurred during enzyme treatment of the pectic substances at pH 5.5 for 96 hours at 20° C in an aqueous solution. The paper chromatograms were run for 24 hours, using a solvent *n*-amyl alcohol, pyridine, and H<sub>2</sub>O (7:7:6 v/v) at 20° C. The uronic acids on the papers were eluted in H<sub>2</sub>O, then evaporated to dryness under vacuum at 20° C.

**Procedure:** Samples of 3, 5, 10, 15 and 20 mg of non-mutarotated  $\alpha$ -D-galacturonic were placed in 1 dram stoppered bottles, using same procedure also for dry-crystalline  $\beta$ -D-glucuronic acid. To each was added 1 ml of the pyridine-silane mixture. The bottles containing 15 and 20 mg of the two standard uronides were warmed slightly to aid solubility. After 10 minutes, 2  $\mu$ l of the reaction mixture was injected directly into the instrument. Mutarotated  $\alpha$ -D-galacturonic and  $\beta$ -D-glucuronic acids were treated similarly. The same procedure was followed for arabinose and glucose, using concentrations of 5 mg/ml. Samples of 2–5  $\mu$ l of the hexuronic acids from apple tissue were also warmed prior to injections.

In the development of this technique, several problems were encountered. A film thought to be silicon dioxide was deposited on the flame detector. To avoid this, detectors were cleaned in warm H<sub>2</sub>O after 10 injections and injections were kept at a minimum volume of 1 and 2  $\mu$ l for best results. Alexander and Garbutt (1) have suggested sorbitol as an internal standard for the determination of D-glucose by the TMS derivative procedure. This may be needed for quantitative determinations of galacturonic and glucuronic acids and their anomers. The other problem of sample size at time of injection was aided by use of a 5  $\mu$ l Hamilton micro-syringe. Two  $\mu$ l injections were easily duplicated. Reaction mixtures were stable and could be measured after standing at room temperature for 10 minutes or after 4–5 hours with the same results.

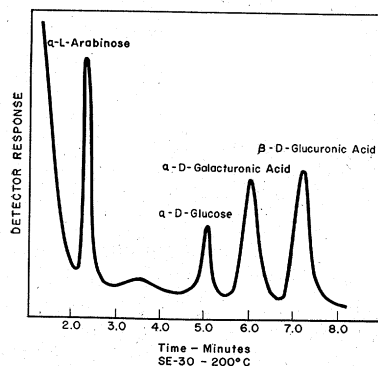


Fig. 1. TMS derivatives of arabinose, glucose, galacturonic acid and glucuronic acid.

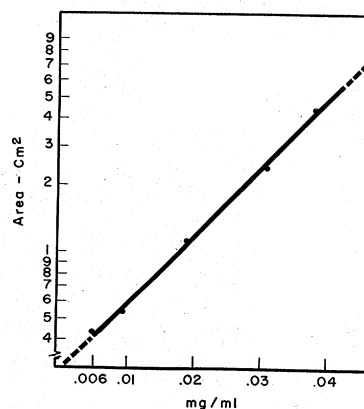


Fig. 2. Standard curve to determine concentrations of galacturonic and glucuronic acids TMS derivatives.

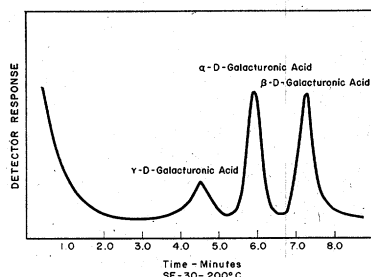


Fig. 3. TMS derivatives of  $\alpha$ -D-galacturonic acid, mutarotated at pH 5.5.

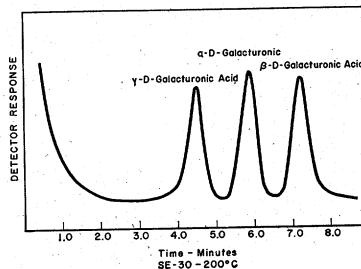


Fig. 4. TMS derivatives of  $\alpha$ -D-galacturonic acid, mutarotated at pH 3.2.

## RESULTS

Treating non-mutarotated hexuronic acids to form methyl esters from the free carboxyl groups prior to the addition of the pyridine-silane solution was not necessary. The COOH groups were not a problem in gas chromatography as in paper and thin layer chromatography. The dry pyridine in the mixture appears to stop mutarotation.

Fig. 1 shows the separation of a mixture of  $\alpha$ -L-arabinose,  $\alpha$ -D-glucose,  $\alpha$ -D-galacturonic acid and  $\beta$ -D-glucuronic acid TMS derivatives in which the carbohydrates were in concentrations of approximately 5 mg/ml. The retention times were *ca* 2.2, 5.0, 5.9, and 7.1 minutes for arabinose, glucose, galacturonic and glucuronic acids, respectively. The peaks of each sugar and sugar acid were well defined and adequately separated for exact identification and quantification. The tailing of the pyridine was not a problem when 1 or 2  $\mu$ l of the pyridine-silane mixture was injected.

Since  $\alpha$ -D-galacturonic and  $\beta$ -D-glucuronic acid TMS derivatives showed good separation at relatively short retention times, the uronic acid concentrations for the derivatives, expressed as area under the curve, were converted to log values and plotted against known concentrations of the uronic acid in the pyridine-silane mixture. This transformation of the data provided a straight line relationship between the 2 sets of figures. The data in Fig. 2 are based on an instrument attenuation of  $\times 32$ . Each point represents the average area of 3 injections. The slope and position of the curve is identical for the 2 hexuronic acids. Difference in retention time represented the separation of these 2 closely related acids.

Fig. 3 shows the separation of mutarotated  $\alpha$ -D-galacturonic acid TMS derivatives into 3 anomers at pH 5.5, 20° C and in H<sub>2</sub>O for 24 hours. The peak at 5.9 min is identified as  $\alpha$ -D-galacturonic acid; at 7.3 min  $\beta$ -D-galacturonic acid; at 4.5 min tentatively identified as  $\gamma$ -D-galacturonic acid which is probably in the furanose form. Similar treatment at the pH of galacturonic acid shows equal amounts of the 3 anomers (Fig. 4).

The separation of mutarotated  $\beta$ -D-glucuronic acid TMS derivatives into 2 anomers at pH 5.5, 20° C and in H<sub>2</sub>O for 24 hours, is

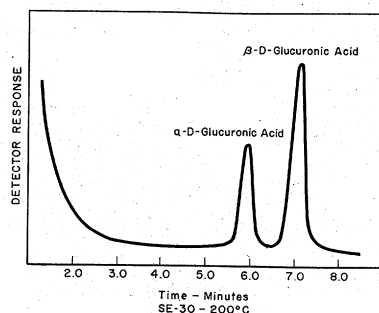


Fig. 5. TMS derivatives of  $\beta$ -D-glucuronic acid, mutarotated at pH 5.5.

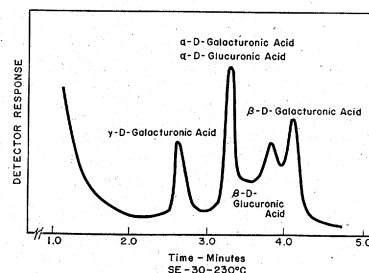


Fig. 6. TMS derivatives of a mixture of  $\alpha$ -D-galacturonic and  $\beta$ -D-glucuronic acids, mutarotated at pH 5.5.

shown in Fig. 5. The peak at 7.1 min is identified as  $\beta$ -D-glucuronic acid, at 5.9 min  $\alpha$ -D-glucuronic acid. Similar treatment at the pH of glucuronic acid showed the same anomers in the 1-1 ratio.

Separation of mutarotated mixtures of the hexuronic acids TMS derivatives is shown in Fig. 6. Only 4 anomers can be separated, with  $\alpha$ -D-galacturonic and  $\beta$ -D-glucuronic acid coming out together on the SE-30 column, although oven temperature was raised to 230°C.

The anomers of hexuronic acids derived from apple fruit cell-walls is shown in Fig. 7. Mutarotation is assumed to have occurred at pH 5.5 during enzyme hydrolysis of the pectin precipitate. The 3 anomers ( $\gamma$ -D,  $\alpha$ -D, and  $\beta$ -D-galacturonic acids) appear identical to those found in Fig. 3 for mutarotated  $\alpha$ -D-galacturonic acid.

#### DISCUSSION

Sweeley and Walker (8) stated that it should theoretically be possible to separate TMS derivatives of mutarotated glucose and galactose mixtures on a nonpolar column. They indicated, however, that the separation was difficult to achieve and that only columns with exceptionally high efficiencies were satisfactory. The use of TMS methyl glycosides of glucose and galactose obtained after methanolysis for separation on either SE-30 or SE-52 columns was recommended.

In the present work, anomers of mutarotated D-galacturonic and D-glucuronic acids were easily separated. Mixtures of mu-

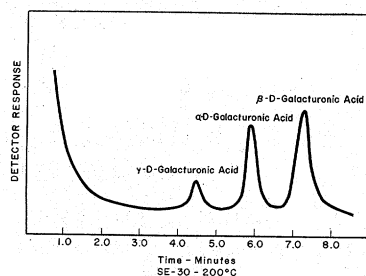


Fig. 7. TMS derivatives of hexuronic acids hydrolyzed from apple fruit.

tarotated galacturonic and glucuronic acids however were not fully separated on the SE-30 columns. The alpha anomers of each were not separated while the beta anomers separated at 230° C. The substance referred to as  $\gamma$ -D-galacturonic acid is thought to be in the furanose form. Disregarding anomerization, the only difference between D-galacturonic and D-glucuronic acids is the position of the OH group on C 4. It is obvious as determined in this study, that the same anomers of each acid, i.e.,  $\alpha$  and  $\alpha$ , are more nearly alike in structure than the anomers of the individual acids, i.e.,  $\alpha$  and  $\beta$ .

Tavakoli and Wiley (10) have reported the possibility of glucuronic acid occurring in apple pectic substances. The present work shows that the glucuronic acid reported by them is probably  $\beta$ -D-galacturonic acid. Measurement of hexuronic acids from apple tissue does not appear to present the additional complication of also measuring the anomers of D-glucuronic acid. Concentrations of anomers of D-galacturonic acid can be determined from the data in Fig. 2 or total D-galacturonic acid can be determined by summing the areas under the curves of  $\alpha$ ,  $\beta$  and  $\gamma$ -D-galacturonic acid.

Apple pectins have also been reported to contain pentosans and hexosans (10), also referred to as "ballast" materials. Fig. 1 shows the separation of several monomers of these materials from the hexuronic acids. Since anomerization is almost impossible to avoid in removing these substances from the parent polysaccharide, a preliminary step using paper chromatography to separate the hydrolytic products into hexuronic acids, aldohexoses and aldopentoses is probably necessary. Each distinct group can be eluted, dried and then treated with the pyridine-silane mixture.

#### LITERATURE CITED

1. ALEXANDER, R. J. and J. T. GARBUTT. 1965. Use of sorbitol as internal standard in determination of D-glucose by gas liquid chromatography. *Anal. Chem.* 37:303-305.
2. ASH, A. S. F. and REYNOLDS, T. M. 1954. Some observations on the occurrence of free galacturonic acid in fruit. *Aust. J. of Bio. Sci.* 7:435-443.
3. BENTLEY, R., C. C. SWEELEY, M. MAKITA, and W. W. WELLS. 1963. Gas chromatography of sugars and other polyhydroxy compounds. *Biochem. Biophys. Res. Commun.* 11:14-18.
4. FERRIER, R. J., and M. F. SINGLETON, 1962. Trimethylsilyl of D-xylose and the nuclear magnetic resonance spectra of the major products. *Tetrahedron* 18:1143-8.
5. HEDGLEY, E. J. and W. G. OVEREND. 1960. Trimethylsilyl derivatives of carbohydrates. *Chem. and Ind. (London)* 378-380.
6. HELBUT, J. R. and K. D. BROWN. 1961. Identification of aldohexuronic acids free and in polymers. *Anal. Chem.* 33:1610-1613.
7. MCINNES, A. G., D. H. BALL, F. P. COOPER, and C. T. BISHOP. 1958. Separation of carbohydrate derivatives by gas-liquid partition chromatography. *J. Chromatog.* 1:556-557.
8. SWEELEY, C. C. and BARBARA WALKER. 1964. Determination of carbohydrates in glycolipides and gangliosides by gas chromatography. *Anal. Chem.* 36:1461-1466.
9. ———, R. BENTLEY, M. MAKITA, and W. W. WELLS. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Amer. Chem. Soc.* 85:2497-2507.
10. TAVAKOLI, M. and R. C. WILEY. 1965. Qualitative separation of enzymatic degradation products obtained from apple cell wall polysaccharides. *Proc. Amer. Soc. Hort. Sci.* 87:104-112.